In vitro induction of organic anion transporting polypeptides (OATPs) as a possible mechanism for thyroid hormone disruption by hexabromocyclododecane (HBCD)

Jorke H. Kamstra[1], Tingting Du[123], Theo J. Visser[4], Leo T.M. van der Ven[2], Albertinka J. Murk[3], Timo Hamers[1]

[1]Vrije Universiteit Amsterdam - Institute for Environmental Studies (IVM), De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands
[2]National Institute for Public Health and the Environment (RIVM), PO Box 1, 3720 BA Bilthoven, The Netherlands
[3]Wageningen University – Toxicology Group, Tuinlaan 5, 6703 HE Wageningen, The Netherlands
[4]Erasmus University Medical Center - Dept of Internal Medicine, Dr Molewaterplein 50, 3015 GE Rotterdam, The Netherlands

Introduction

Hexabromocyclododecane (HBCD) is a brominated flame retardant (BFR), which in terms of global production volumes has a third position after tetrabromobisphenol-A and polybrominated diphenyl ethers. HBCD is used in polymer and textiles industries as well as insulation panels and blocks for building constructions. Commercial technical mixtures of HBCD consist of three diastereoisomers (alpha, beta, and gamma) of which the gamma diastereoisomer typically contributes for 70-90% to the composition of the mixture. Behavioural studies on mice neonatally exposed to HBCD showed that motor behaviour, learning and memory processes were affected (Eriksson et al. 2006), suggesting that HBCD could disrupt thyroid hormone system. In a recently published 28-day oral dose experiment with HBCD in rats, decreased plasma levels of thyroxin [T4] were reported in females, with concomitant increased pituitary weight, TSH immunostaining in the pituitary, thyroid weight, and thyroid follicle cell activation (Van der Ven et al. 2006). In the present study, we investigate the hypothesis that an increase in tissue T4 uptake and metabolism may be one of the underlying mechanisms for decreased plasma levels. Increased cellular TH uptake was also suggested by results from the in vitro T-Screen bioassay, a TH-dependent proliferation assay with rat pituitary cells. T-Screen results indicated that HBCD itself is not able to induce cell proliferation, whereas HBCD significantly enhances 3,3',5-triiodothyronine (T3)-induced cell proliferation (Hamers et al. 2006; Schriks et al. 2006; Yamada-Okabe et al. 2005). Organic anion transporting polypeptides (OATPs) are a large family of homologous membrane bound solute carrier proteins (Hagenbuch and Meijer, 2004), of which many can transport iodothyronines. In rat, T4 and T3 transporting isoforms OATP1A1 and OATP1A4 are expressed in the liver (Friesema et al., 2005). In this study, OATP1A1 and OATP1A4 expression was determined in rat liver carcinoma H4IIE cells exposed to HBCD by real-time quantitative polymerase chain reaction (RT-qPCR).

Materials and Methods

A technical mixture of HBCD was obtained from Dead Sea Bromine Group. Reference compounds pregnenolone-16α-carbonitrole (PCN) and 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) were purchased from Sigma (St. Louis MO USA) and DMSO from Acros Organics (New Jersey, USA). All test chemicals were dissolved in DMSO. H4IIE cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C in αMEM medium, supplemented with 10% fetal calf serum (Gibco, Auckland, NZ). Cells were seeded on 6-well plates at a density of 1.5×10^5 cells/ml in a total volume of 3 ml and incubated overnight. Cells were subsequently exposed to HBCD (0.1-10μM), PCN (0.1-1μM), TCPOBOP (0.25-2.5 μM), or a carrier control (0.1% DMSO). After 24h of exposure,

medium was removed, cells were lysed, and total RNA was extracted using the NucleoSpin RNA II kit (Macherey-Nagel, Germany). Total RNA was quantified spectrophotometrically in a Nanodrop analyser (ND-1000, Wilmington, USA) and adjusted to similar concentrations with RNAse free water. RNA integrity was analyzed by agarose gel electrophoresis. Around 800 ng total RNA was reverse transcribed with the iScript-Kit from Bio-Rad (Hercules, USA) with a final volume of 20 µl. cDNA samples were diluted 20 times with sterile water and stored at -20ºC until further analysis. RT-qPCR analysis was performed with the My iQ Real-Time PCR Detection System and My iQ Software (Bio-Rad, Hercules, USA). Samples were assayed in triplicate in 20 µl reaction mixture containing 2x iQ SYBR Green Supermix (Bio-Rad), 5 µl cDNA per reaction, and gene specific primers (250 nM), which were checked for efficiency and dimer formation prior to analysis. The cycling program was: denaturation (95ºC for 3 min), two-step amplification and quantification (95ºC for 15 sec and 60ºC for 45 sec with a single fluorescent measurement). After 40 cycles, a melting curve was generated (60-90ºC, 0.5ºC per 10 sec) to check for multiple products.

Results and Discussion

For OATP1A4, a significant and dose-dependent increase in mRNA expression levels was found in H4IIE cells exposed to HBCD (Table 1). A similar OATP1A4 induction was found for the PCN, which is a typical activator of the rodent pregnane-X receptor (PXR). For TCPOBOP, a typical activator of the rodent constitutive androstane receptor (CAR), OATP1A4 induction was not significant (Table 1). These results suggest that up-regulation of OATP1A4 in HBCD exposed rat H4IIE cells should be attributed to PXR activation. In vivo studies, however, have demonstrated that OATP1A4 expression could be upregulated through both PXR and CAR in mice (Wagner et al. 2005, Staudinger et al. 2003). In addition, elevated levels of CYP3A1/3 and CYP2B1 were found in HBCD exposed rat livers suggesting that HBCD is a ligand for PXR and CAR, respectively (Germer et al. 2006). The role of CAR-activation in HBCD induced OATP1A4 expression in H4IIE cells cannot be confirmed in the in vitro RT-qPCR experiments, possibly because this signaling pathway is lost in the H4IIE in vitro cell line.

Table 1: OATP1A4 mRNA-expression levels in H4IIE cells exposed to different test compounds. Significant differences from control are indicated by asterisk ($p<0.05$).

Exposure	Concentration (µM)	Induction factor
Control (0.1% DMSO)	-	1.0
HBCD	0.1	0.94
	1.0	1.6
	10	2.4*
PCN	0.1	1.3
	1.0	2.1*
TCPOBOP	0.25	1.3
	2.5	1.4

For OATP1A1, constitutively expressed mRNA levels were too low for quantification. Also after exposure to test compounds HBCD, PCN, and TCPOBOP no quantifiable response was achieved. These results are consistent with in vivo studies in which no OATP1A1 induction was found in PCN exposed rats (Rausch-Derra et al. 2001) and mice (Wagner et al. 2005).

Results so far confirm that HBCD exposure leads to upregulation of OATP1A4 expression. Additional experiments are necessary, however, to support the hypothesis that induced OATP1A4 expression leads to increased cellular TH uptake and consequently to decreased T4 plasma levels in HBCD exposed rats. To confirm that OATP1A4 is also induced in vivo, additional RT-qPCR experiments are planned on rat liver mRNA from the same HBCD-exposed rats, for which Van der Ven et al. (2006) reported decreased T4 levels in plasma. To confirm that HBCD stimulates cellular TH uptake, preliminary TH uptake experiments were performed in H4IIE cells using immunostaining techniques. Results from these experiments suggested induction of TH uptake by HBCD. Currently, additional uptake experiments are performed with radiolabeled ^{125}I-T4. Results from in vivo OATP1A4 RT-qPCR measurements and in vitro TH uptake experiments will be presented at BFR2007. So far, the combination of gene expression analysis and immunocytochemistry suggest that increased cellular TH-uptake is a target of HBCD, and may be a novel mechanism by which the in vivo effects of this BFR are evoked.

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